Plant Foods for Human Nutrition

Cocoa flavanols protect human endothelial cells from oxidative stress

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Abstract:	Oxidative stress may cause functional disorders of vascular endothelia which can lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues. Plant antioxidants protect the endothelium against oxidative stress and then become an effective option to treat vascular diseases. Cocoa flavanols have been proved to protect against oxidative stress in cell culture and animal models. In addition, epidemiological and interventional studies strongly suggest that cocoa consumption has numerous beneficial effects on cardiovascular health. The objective of this study was to test the chemo-protective effect of realistic concentrations of a cocoa phenolic extract and its main monomeric flavanol epicatechin on cultured human endothelial cells submitted to an oxidative challenge. Both products efficiently restrained stress-induced reactive oxygen species and biomarkers of oxidative stress such as carbonyl groups and malondialdehyde, and recovered depleted glutathione, antioxidant defences and cell viability. Our results demonstrate for the first time that a polyphenolic extract from cocoa and its main flavonoid protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and antioxidant enzyme and non-enzyme defences.
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Response to Reviewers:	

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

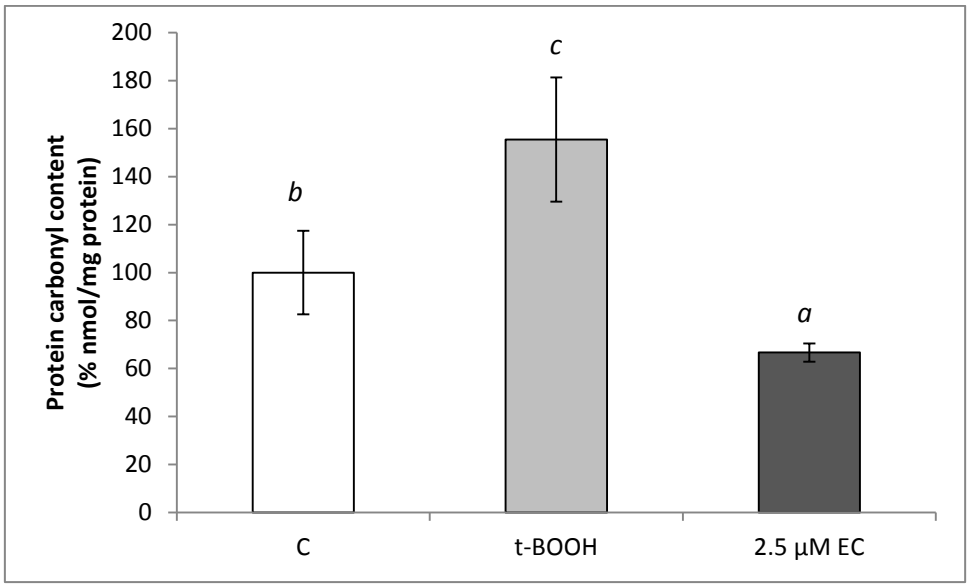
Abstract

Oxidative stress may cause functional disorders of vascular endothelia which can lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues. Plant antioxidants protect the endothelium against oxidative stress and then become an effective option to treat vascular diseases. Cocoa flavanols have been proven to protect against oxidative stress in cell culture and animal models. In addition, epidemiological and interventional studies strongly suggest that cocoa consumption has numerous beneficial effects on cardiovascular health. The objective of this study was to test the chemo-protective effect of realistic concentrations of a cocoa phenolic extract and its main monomeric flavanol epicatechin on cultured human endothelial cells submitted to an oxidative challenge. Both products efficiently restrained stress-induced reactive oxygen species and biomarkers of oxidative stress such as carbonyl groups and malondialdehyde, and recovered depleted glutathione, antioxidant defences and cell viability. Our results demonstrate for the first time that a polyphenolic extract from cocoa and its main flavonoid protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and antioxidant enzyme and non-enzyme defences.

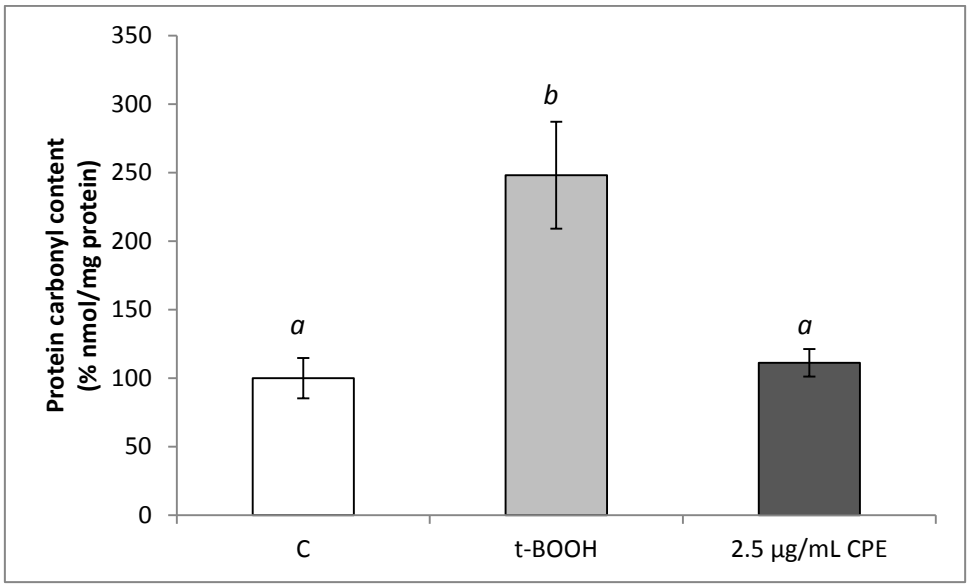
Supplementary table 1.- Peak identification and compound content of the CPE

Compound	Peak No	Rt (min)	mg/100g
Theobromine	1	6.6	891.1
Procyanidin B1	2	7.7	34.9
Catechin	3	9.7	116.9
Procyanidin B2	4	11.7	133.2
Epicatechin	5	13.3	383.5
Procyanidin (trimer)	6	15.6	62.4
Procyanidin (dimer)	7	23.3	24.0
Total flavanols			*754.9*

A)



B)



Answers to reviewers

We wish to thank the reviewers for the careful reading of the manuscript and encouraging comments, as well as their interesting queries and suggestions.

Replies to comments from reviewer 1:

-I think that the composition of the extract should be also presented, although it is presented in the other paper.

Reply: in agreement with the reviewer the precise composition of the extract has been included in the revised version as supplementary table 1.

-Did the authors use the same cocoa extract as in the paper from the [11] position? If it is so, how was it stored? Hasn't it changed its properties? The previous research was published quite a long time ago (11 years). Or was a cocoa bought from the same brand more recently and the authors assume that the properties haven't changed? I would be cautious with such statements, since many factors could influence phenolic composition.

Reply: we agree with the reviewer concern about possible loses of phenolic compounds and antioxidant capacity of CPE through time. Indeed the cocoa phenolic extract utilized in the present study is an aliquot from the same extract prepared back in 2008, when aliquots were kept frozen at -20 degrees until assay. However, aliquots of that same extract have been continuously used for years in other studies as close as 2015 (see articles below) and they have shown a remarkable chemo-protective activity; therefore, we are very confident that the frozen extract retains most of its antioxidant capacity, if not all.

Martín et al. 2010. Cocoa flavonoids up-regulate antioxidant enzymes activity via ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. J. Nutritional Biochem. 21: 196-205.

Rodríguez-Ramiro et al. 2011. Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. J. Nutr. Biochem. 22: 1186-1194.

Rodríguez-Ramiro et al. 2013. Cocoa polyphenols prevent inflammation in the colon of azoxymethane-treated rats and in TNF-α-stimulated Caco-2 cells. Brit. J. Nutr. 110: 206-215.

Martín et al. 2013. Cocoa phenolic extract protects pancreatic beta cell viability and function against oxidative stress. Nutrients 5: 2955-2968.

Cordero-Herrera et al. 2014. Cocoa flavonoids attenuate high glucose-induced insulin signalling blockade and modulate glucose uptake and production in human HepG2 cells. Food Chem. Toxicol. 64:10-19.

Cordero-Herrera et al. 2015. Cocoa flavonoids protect hepatic cells against high glucose-induced oxidative stress: Relevance of MAPKs. Mol. Nutr. Food Res. 59: 597-609

-What was the epicatechin concentration in the cocoa flavanols extract? It should be written in the manuscript. Was it at the same level as in the solution with epicatechin only?

Reply: we absolutely agree with the comment; a comprehensive explanation of the EC concentration and its relative amount in CPE has now been included in the text in page 6, lines 139-140. Actual concentrations of epicatechin in tested doses of CPE ranged from 33 nM in the dose of 2.5 ug CPE/mL to 0.265 uM in that of 20 ug CPE/mL.

-Have authors searched for the resveratrol in this cocoa flavanol extract? Some researches e.g. Counet, C.; Callemien, D.; Collin, S. Chocolate and cocoa: New sources of trans-resveratrol and trans-piceid. Food Chem. 2006, 98, 649-657 state that it can be found in cocoa and many researches confirm its protective properties for cardiovascular system.

Reply: I honestly was not aware of that finding and I thank the reviewer for the information. We knew that, besides flavanols, three flavonols, isoquercitrin, quercetin 3-glucuronide and quercetin, had been found in cocoa powder using HPLC-DAD coupled with HPLCMS (Lamuela-Raventós et al. J Nutr 2001, 131:834-835). However, cocoa powder contains as much as 30 mg flavonols/100 g and only 0.4 ppm of trans-resveratrol (Counet et al. above) whereas over 755 mg/100 g of flavanols, thus we believe that most of the chemo-preventive potential of cocoa is due to flavanols.

-what about the influence on the endothelium of the other phenolic compounds found in the cocoa extract?

Reply: Influence on endothelium of other major compounds such as theobromine cannot be ruled out but we also should consider that most of the CPE-derived chemo-protective effects described in this study are also observed when epicatechin is tested alone, that is why we hypothesized that the majority of the protective effect resulted from the effect of epicatechin with the help of other flavanols. In any case, and in agreement with the comment of the reviewer, we have added a short sentence in page 9, lines 206-207, indicating that the potential effect of other compounds besides EC should not be ruled out.

Replies to comments from reviewer 2:

Reviewer #2: The authors present their work on the antioxidant action of cocoa polyphenols on cultured human endothelial cells submitted to an oxidative challenge. The work is interesting, however, few points should be addressed before the publication. List of abbreviations should be added in the manuscript, also, in figure legends full names to explain abbreviations (as in the text where they are mentioned the first time) are strongly suggested.

Reply: we are sorry we have not included a list of abbreviations in the revised version of the manuscript but, due to format restrictions, addition of a list of abbreviations would have exceeded the size of the document over the maximum of 16 pages allowed by the journal requirements. We kindly ask the reviewer to ponder that reduction of the initial document to less than 50 % has implied a considerable effort by the authors including removal of 16 references and transferring two tables and 1 figure to supplementary material. However, following the reviewer suggestion we have made sure that all acronyms have been spelled out at first appearance in the text and all acronyms have also been spelled out in figure legends.

For the complete discussion about the antioxidant action of cocoa polyphenols, could the authors explain why they have not analyzed the effects of cocoa polyphenols on two main antioxidative enzymes in the cells - catalase (CAT) and superoxide dismutase (SOD)?

Reply: indeed activity of catalase and superoxide dismutase have been determined previously in most of our studies dealing with chemo-protective effect of plant antioxidants against oxidative stress but lately we stopped evaluating CAT due to its large Km and low specific affinity, particularly in endothelial cells. On the other hand, SOD is a solid biomarker of the cell redox status that we have repeatedly analyzed in previous studies when inconsistent results were obtained with glutathione peroxidase and glutathione reductase in order to confirm the cellular antioxidant response to face a condition of oxidative stress. However, since in the present study the response of the antioxidant defenses was clear we decided that it was not necessary to spend over 600 euros for the SOD assay kit. We feel that the comprehensive response of the two defense enzymes analyzed support the hypothesis of the chemo-preventive effect of cocoa flavanols on endothelial cells submitted to oxidative stress.

Replies to comments from reviewer 3:

Reviewer #3: 33 proved proven

47 of nowadays nowadays of

Reply: both corrected in lines 34 and 51 of revised version.

85 It should be mentioned if correct that analyses of cell redox status and cell oxidative damage and cell protein were performed on cell lysates. Also the method to prepare the lysates should be described.

Reply: as suggested by the reviewer it has been stated in page 5, lines 117-118 that analyses of cell redox status and cell oxidative damage and cell protein were performed on cell lysates. Since cell lysate for each assay is prepared following a different protocol we have been unable to include all particular methods of preparation. As with the rest of referees we kindly ask the reviewer to consider that reduction of the initial document to less than 50 % has implied a considerable effort by the authors including removal of interesting information plus 16 references and transferring two tables and 1 figure to supplementary material; thus, addition of specific information for each lysate preparation would result in exceeding the maximum length of the document.

161 resulted in significantly …. Also list p value for this comparison.

Reply: in order to fulfil the size requirement of the journal a large amount of text has been removed from the original version, including the sentence that you suggest to correct.

179 In the discussion the authors should data from the analysis of the cocoa extract such as concentration of monomers and dimers the only components capable of entering the bloodstream and getting to the cells. This will give some uM comparison to your EC data.

Reply: following your advice and that of other reviewer a new table 1 depicting the cocoa extract composition has been included as supplementary material.

192 human consumption, 50 g? of chocolate

Reply: the sentence has been corrected in line 138; indeed it was 50 g of chocolate.

207 a deed an effect

Reply: as above, the sentence has been removed.

237 live life

Reply: corrected in line 199 of revised version.

Cocoa flavanols protect human endothelial cells from oxidative stress

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Abstract

Oxidative stress may cause functional disorders of vascular endothelia which can lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues. Plant antioxidants protect the endothelium against oxidative stress and then become an effective option to treat vascular diseases. Cocoa flavanols have been proven to protect against oxidative stress in cell culture and animal models. In addition, epidemiological and interventional studies strongly suggest that cocoa consumption has numerous beneficial effects on cardiovascular health. The objective of this study was to test the chemo-protective effect of realistic concentrations of a cocoa phenolic extract and its main monomeric flavanol epicatechin on cultured human endothelial cells submitted to an oxidative challenge. Both products efficiently restrained stress-induced reactive oxygen species and biomarkers of oxidative stress such as carbonyl groups and malondialdehyde, and recovered depleted glutathione, antioxidant defences and cell viability. Our results demonstrate for the first time that a polyphenolic extract from cocoa and its main flavonoid protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and antioxidant enzyme and non-enzyme defences.

Keywords: epicatechin**,** catechins, procyanidins, EA.hy926 cells, chocolate flavanols, endothelial dysfunction.

Funding: this work was supported by the grant AGL 2015–67087-R (MINECO/FEDER, UE) from the Spanish Ministry of Science and Innovation.

Conflict of interest: the authors declare that they have no conflict of interest.

Availability of data and material (data transparency): Not applicable

Code availability (software application or custom code); Not applicable

Authors' contributions: TFM and LG carried out most of the experimental and data analysis. OP carried out and analyzed MDA assay. SR carried out and analyzed data from carbonyl groups. DAC and MAM contributed to the critical revision of the manuscript. LG conceived and designed the study and wrote the manuscript with significant contributions from all other authors.

Abbreviations list

CPE: cocoa polyphenolic extract

CVD: cardiovascular disease

DCF: 2′,7′-dichlorofluorescein

DCFH: 2′,7′-dichlorohydrofluorescein

DMEM: Dulbecco's modified eagle's medium

DMSO: dimethyl sulfoxide

DNPH: dinitrophenylhydrazone

EC: epicatechin

FBS: foetal bovine serum

GPx: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

LDL: low density lipoprotein

MDA: malondialdehyde

NADPH: nicotine adenine dinucleotide phosphate reduced salt

NO: nitric oxide

OPT: o- phthaldialdehyde

ROS: reactive oxygen species

t-BOOH: tert-butylhydroperoxide

TEP: 1,1,3,3-tetraethoxypropane

Introduction

Oxidative stress seems to be the common underlying mechanism for the development of endothelial dysfunction; when reactive oxygen species (ROS) production is not balanced by antioxidant defense systems, the generated oxidative stress may cause functional disorders of vascular endothelia which can

lead to endothelial apoptosis and thus alter the function and structure of the vascular tissues [1]. Therefore, prevention of oxidative stress is one of the main objectives nowadays of cardiovascular research and consequently, considerable efforts have been made in the last years for the identification of natural antioxidants and dietary products containing these bioactive compounds, which may provide valuable strategies to prevent oxidative stress and the development of cardiovascular disease (CVD).

Polyphenols are some of the most abundant phytochemicals in plant foods and increasing evidence from cohort studies indicate that the intake of some of these compounds may help to reduce the development of CVDs and CVDs mortality risk [2]. Polyphenols encompass several families of compounds, the most represented in plant foods being phenolic acids and flavonoids [3]. A major group of flavonoids is constituted by flavanols that are abundant in green tea, red wine, cocoa and various fruits such as apples [4]. Among them, cocoa beans are one of the richest known sources of flavonoids; indeed, cocoa is the food that has the highest flavonoid content on a per-weight basis [4,5]. The main constituents are flavanols, present as monomeric (−)-epicatechin (EC) and (+)-catechin, together with their oligomers, the so-called proanthocyanidins, responsible for cocoa bitterness. Epidemiological and interventional studies strongly suggest that cocoa consumption, as well as vegetables and fruit intake, has numerous beneficial effects on cardiovascular health [6] including an improvement in vascular function [2,7]. Various potential mechanisms, including the increased bioavailability of NO [8] and the anti-inflammatory and antioxidant effect [9], are supposed to be responsible for the protective properties of cocoa. However, scarce research is available on the intrinsic mechanisms involved in such effects at cellular level. Therefore, the objective of this study was to test the chemo-protective effect of a cocoa phenolic extract (CPE) and its main monomeric flavanol EC on endothelial cells submitted to an oxidative challenge. To this purpose, a human endothelial cell line, EA.hy926, was used as a cell culture model of endothelium and treatment with a strong pro-oxidant, *tert*-butylhydroperoxide (t-BOOH), was used to reproduce a cell culture condition of oxidative stress in

order to study the possible protective mechanisms through which cocoa flavanols protect endothelial function.

Materials and methods

Reagents and materials

Tert-butylhydroperoxide (t-BOOH), (-)-epicatechin (>95% of purity), glutathione reductase (GR), reduced (GSH) and oxidized glutathione, dichlorofluorescin (DCFH), o-phthaldialdehyde (OPT), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2,4-dinitrophenylhydrazine (DNPH), H2O2, 1,1,3,3-tetraethoxypropane (TEP), gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical Co. (Madrid, Spain). Acetonitrile, methanol of HPLC grade, dimethyl sulfoxide (DMSO) were acquired from Panreac (Barcelona, Spain). Bradford reagent was from BioRad Laboratories S.A. DMEM culture media and foetal bovine serum (FBS) were from Cultek (Madrid, Spain). All other reagents were of analytical quality.

Sample preparation

Natural Forastero cocoa powder (Idilia Foods, Barcelona, Spain) was used to prepare the CPE. Extraction of soluble polyphenols and characterization of the different components by HPLC has been detailed elsewhere [10]. Briefly, the polyphenolic profile of CPE showed that monomeric EC and catechin were the major flavanols in the extract, together with appreciable amounts of procyanidins B1 and B2. Additionally, theobromine was present in high amounts while only traces of caffeine were detected in the extract (supplementary data, table 1).

Cell culture and treatment

EA.hy926, a human hybrid cell line, was a kind gift from Profs. Patricio Aller and Carmelo Bernabeu, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain. The cell line was cultured and passaged in Biowhittaker DMEM media supplemented with 10 % fetal bovine serum. Cells were maintained in a humidified incubator containing 5 % CO2 and 95 % air at 37 °C and grown in DMEM medium supplemented with 10 % FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin

and streptomycin [11]. Different concentrations of CPE (2.5, 5, 10 and 20 µg/mL) and EC (2.5, 5, 10 and 20 µM), dissolved in serum-free culture medium, were added to the cell plates for 18 hours to study a direct/basal effect of the cocoa extract and its main phenolic compound. In order to evaluate the protective effect of the CPE and EC against an oxidative insult, two different approaches were carried out, co-treatment and pre-treatment. In the co-treatment assay EA.hy926 cells were simultaneously treated for 18 h with 100 µM t-BOOH plus any of the four different CPE or EC concentrations, whereas in the pre-treatment assay cells were first treated with tested doses of CPE or EC for 18 h, then washed and submitted to a new media containing 200 µM t-BOOH for 4 h.

Determination of cell redox status, biomarkers of oxidative damage and cell viability

Cellular ROS were quantified by the DCFH assay using microplate reader and the assay has been described elsewhere [10,11]. GSH was quantitated by the fluorometric assay described in Browne & Armstrong [12] with some modifications. Assay of GPx activity is based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR as described by Martín et al. [10]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [10]. Malondialdehyde (MDA) was analyzed by high-performance liquid chromatography (HPLC) as its DNPH derivative [10], measured in an Agilent 1100 Series HPLC-DAD. MDA values are expressed as nmol of MDA/mg protein. Protein oxidation of cells was measured as carbonyl groups content according to a published method [11]. Analyses of GSH, GPx, GR, MDA and carbonyl groups were performed on cell lysates. Protein was measured by the Bradford reagent. Cell viability was determined by using the crystal violet assay [11].

Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a

Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. A SPSS version 23.0 program has been used.

Results and discussion

Biological activities of cocoa flavanols include scavenging of active oxygen species, prevention of LDL oxidation, anti-inflammatory activity, inhibition of tumor cell growth, regulation of apoptotic and survival/proliferation pathways, antidiabetic effects and cardiovascular benefits [1-3,10,13]. All these properties make cocoa polyphenolic fraction an interesting candidate for vascular chemo-protection and, in the present study, the effects of the complex mix of flavanols in CPE have been tested in endothelial cells and the results compared to those with an individual major flavanol EC. The results of the present study demonstrate for the first time that a polyphenolic extract from cocoa powder and its main monomeric flavanol, EC, have the capacity to protect human endothelial cells against an oxidative insult by modulating oxygen radical generation and enzyme and non-enzyme antioxidant defenses.

CPE used in this study has been confirmed as a realistic representative of cocoa-derived products [10,14,15]. Some authors have reported concentrations up to 35 µM of EC in rat serum 1 h after oral administration of EC and 0.2-0.4 µM EC have been detected after consumption of 50 g of chocolate [10]; actual EC concentrations in tested doses of CPE ranged from 33 nM in the dose of 2.5 µg CPE/mL to 0.265 µM in that of 20 µg CPE/mL. Cultured EA.hy926 cells have been recently proved a reliable in vitro model of endothelial tissue [8,11,16] and their viability was not altered by treatment with concentrations up to 20 µg/mL CPE and 20 µM EC for 18 h. When cells were submitted to 100 µM t-BOOH for 18 h or 200 µM t-BOOH for 4 h a dramatic increase in ROS production was observed, but this increase was partially or completely avoided by a co-treatment or pre-treatment with any of the CPE and EC (figure 1). Due to their phenolic structure, flavanols have a remarkable oxidant-scavenging capacity related to the hydrogen donating ability and the stability of the phenoxyl radicals formed [3]. In this study, high levels of ROS generated during the stress period are being more

efficiently quenched in cells co-treated or pre-treated with CPE or EC resulting in a reduced cell oxidative damage. A similar attenuation of ROS has been reported for grape stilbene resveratrol in endothelial cells [17] and analogous cocoa phenolic extracts in hepatic [10] and pancreatic [13] cells.

As the main non-enzymatic antioxidant defense within the cell, it is accepted that GSH depletion reflects a milieu of intracellular oxidation, whereas an increase in GSH concentration places the cell in a more favorable position against a potential oxidative insult [10-12,16]. When cells were submitted to t-BOOH a significant depletion of GSH levels was observed (figure 2C,D), and this alteration was successfully prevented by most of CPE and EC treatments (figure 2), only the highest CPE concentration did not recover the depleted GSH; a fact that has been explained by conjugation of monomeric flavanols to the free sulfhydryl group of reduced glutathione [10]. This protective effect on the cellular antioxidant stores, also observed in endothelial cells treated with plant extracts [11,16], is essential since maintaining GSH concentration above a critical threshold while facing a stressful situation represents an enormous advantage for cell survival.

Activation of GPx and GR is an critical mechanism of the cell antioxidant defense to face oxidative insults by quenching ROS over-production; however, a rapid return of the antioxidant enzyme activities to basal values once the oxidative challenge has been overcome will reestablish favorable conditions for the cell to cope with a new oxidative insult [10,11,16]. Cells submitted to t-BOOH showed a substantial increase of GPx activity as antioxidant response to the oxidative insult, and a full recovery from this increased activity was achieved when cells were co-treated with 5-10 µg/mL of CPE or 5-10 µM EC (figure 3C,D). In the pre-treatment assay, all tested doses of CPE and EC evoked a partial but significant recovery of the stress-enhanced GPx activity (figure 3E,F). A remarkable increase in the activity of GR was observed after treatment with t-BOOH (figure 4C-F), but its activity dose-dependently decreased when cells were co-treated with 2.5-20 µg/mL of CPE (figure 4C) and a full recovery of the GR activity was observed with a co-treatment with EC (figure 4D). A pre-treatment of cells with CPE or EC was able to significantly reduce the stress-enhanced GR activity to values that

were similar to those of control cells. This phenomenon is consistent with previous results with EC and CPE in other cell types [10,13,15]. Hence, while cells submitted to oxidative stress are still fighting to overcome the insult, those treated with the antioxidants have more efficiently controlled the stressful situation and returned to a balanced redox status consequently reducing cell damage.

MDA, a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids, mainly arachidonic acid, is the main product of lipid peroxidation [18] and has been found elevated in several diseases thought to be related to free radical injury; thus, it is widely used as an index of lipo-peroxidation in biomedical sciences [18]. A four-fold increase in MDA concentration was found in endothelial cells after treatment with t-BOOH, but most tested doses of CPE and EC were able to significantly restrain the t-BOOH-induced raise of MDA both in co-treatment and pre-treatment assays (table 1). The significant protection by EC or CPE against an induced lipid peroxidation in endothelial cells is in agreement with previous studies that showed a comparable effect of other compounds [17].

Table 1. Effect of co-treatment and pre-treatment of EA.hy926 cells with EC (2.5, 5, 10 and 20 µM) and CPE (2.5, 5, 10 and 20 µg/mL) on MDA concentration expressed as nmol/mg protein. Results are means ± SD (n=3, 4 replicates). Different letters in each column indicate statistically significant differences ($p < 0.05$) among data.

	nmol MDA/mg prot	
	Co-treatment	**Pre-treatment**
Control	$1.06^{a} \pm 0.21$	$0.69^{b} \pm 0.09$
tBOOH	$3.96^{e} \pm 0.31$	$3.38^{e} \pm 0.53$
2.5 µM EC	$2.75^{c} \pm 0.19$	$0.12^{a} \pm 0.08$
5 µM EC	$2.28^{b} \pm 0.19$	$0.45^{b} \pm 0.02$
10 µM EC	$2.32^{b} \pm 0.41$	$0.67^{b} \pm 0.08$
20 µM EC	$3.70^{d} \pm 0.21$	$1.74^{d} \pm 0.34$
2.5 µg/mL CPE	$2.42^{b} \pm 0.20$	$1.10^{c} \pm 0.12$
5 µg/mL CPE	$2.70^{c} \pm 0.47$	$1.83^{d} \pm 0.35$
10 µg/mL CPE	$3.30^{c,d} \pm 0.11$	$1,.41^{d} \pm 0.05$
20 µg/mL CPE	$3.16^{c} \pm 0.71$	$1.02^{c} \pm 0.10$

Carbonyl groups are considered as consistent biomarkers of oxidative damage to proteins, a crucial event in the development of cellular toxicity [18]. The significant increase in the cellular concentration of carbonyl groups during oxidative stress induced by t-BOOH in cultured endothelial cells confirmed extensive damage to cellular proteins. Nevertheless, the lowest CPE and EC doses tested in this study significantly abolished the protein damage induced by the stressor recovering carbonyl group concentration to values similar to those of control unstressed cells (supplementary data, figure 1). This protective effect against protein oxidation has been previously reported for CPE and EC in pancreatic beta cells [13] and colonic metabolites of flavonoids in endothelial cells [8].

Since both CPE and EC were involved in the regulation of the antioxidant defense mechanisms necessary to face the oxidative challenge, their protective effect should be physiologically exposed in terms of increased cell viability; thus, a potent oxidative challenge induced by t-BOOH severely compromised cell viability but co-treatment or pre-treatment of cells with CPE or EC showed a significant recovery of cell viability in almost all cases (figure 5). Consequently, realistic doses of both antioxidant compounds were capable of preserving cell life and function. Thus, the protective mechanism of CPE and EC on endothelial cells submitted to an oxidative stress could be explained in terms of regulation of the cellular redox status: a decrease of ROS production during stress reduces the need of peroxide detoxification through GPx as well as of GSH and, consequently, its recovery from oxidized glutathione through GR. Furthermore, decreased ROS level reduces oxidative damage to macromolecules, lipids and proteins, resulting in diminished cell injury and death. Interestingly, EC being the most abundant flavanol of cocoa suggests that a great percent of the protecting effect of CPE in endothelial cells may be due to EC, but the potential effect of other compounds besides EC should not be ruled out.

Conclusions

Our studies in cultured endothelial cells demonstrate, for the first time, that flavanol rich extract from cocoa, CPE, and its main flavanol, EC, display positive health effects against an oxidative stress

through an efficient regulation of cell redox status; limiting ROS generation and strengthening antioxidant defense response. The present data propose a prominent role for cocoa and its flavanols in the protection afforded by fruits, vegetables and plant-derived beverages against pathologies such as CVD, for which excessive production of ROS has been implicated as a causal or contributory factor.

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Figure captions

Fig. 1- Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on reactive oxygen species production by EA.hy926 cells. (A,B) direct effect of CPE and EC; (C,D) effect of co-treatment of 100 µM t-BOOH plus noted CPE or EC concentrations for 18 h; (E,F) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 µM t-BOOH. Results are means ± SD (n=3, 4 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data.

Fig. 2 - Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione concentration in EA.hy926 cells. (A,B) direct effect of CPE and EC; (C,D) effect of co-treatment of 100 µM t-BOOH plus noted CPE or EC concentrations for 18 h; (E,F) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 µM t-BOOH. Results are means ± SD (n=3, 4 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data.

Fig. 3 - Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione peroxidase activity in EA.hy926 cells. (A,B) direct effect of CPE and EC; (C,D) effect of co-treatment of 100 µM t-BOOH plus noted CPE or EC concentrations for 18 h; (E,F) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 µM t-BOOH. Results are means ± SD (n=3 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data.

Fig. 4 - Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on glutathione reductase activity in EA.hy926 cells. (A,B) direct effect of CPE and EC; (C,D) effect of co-treatment of 100 µM t-BOOH plus noted CPE or EC concentrations for 18 h; (E,F) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 µM t-BOOH. Results are means ± SD (n=3 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data.

Fig. 5 - Effect of cocoa phenolic extract (CPE) and epicatechin (EC) on EA.hy926 cell viability. (A,B) direct effect of CPE and EC; (C,D) effect of co-treatment of 100 µM t-BOOH plus noted CPE or EC concentrations for 18 h; (E,F) effect of pre-treatment of noted CPE or EC concentrations for 18 followed by 4 h with 200 µM t-BOOH. Results are means ± SD (n=8 replicates). Within each panel, different letters upon data bars indicate significant differences ($p < 0.05$) among data.

Fig. 1

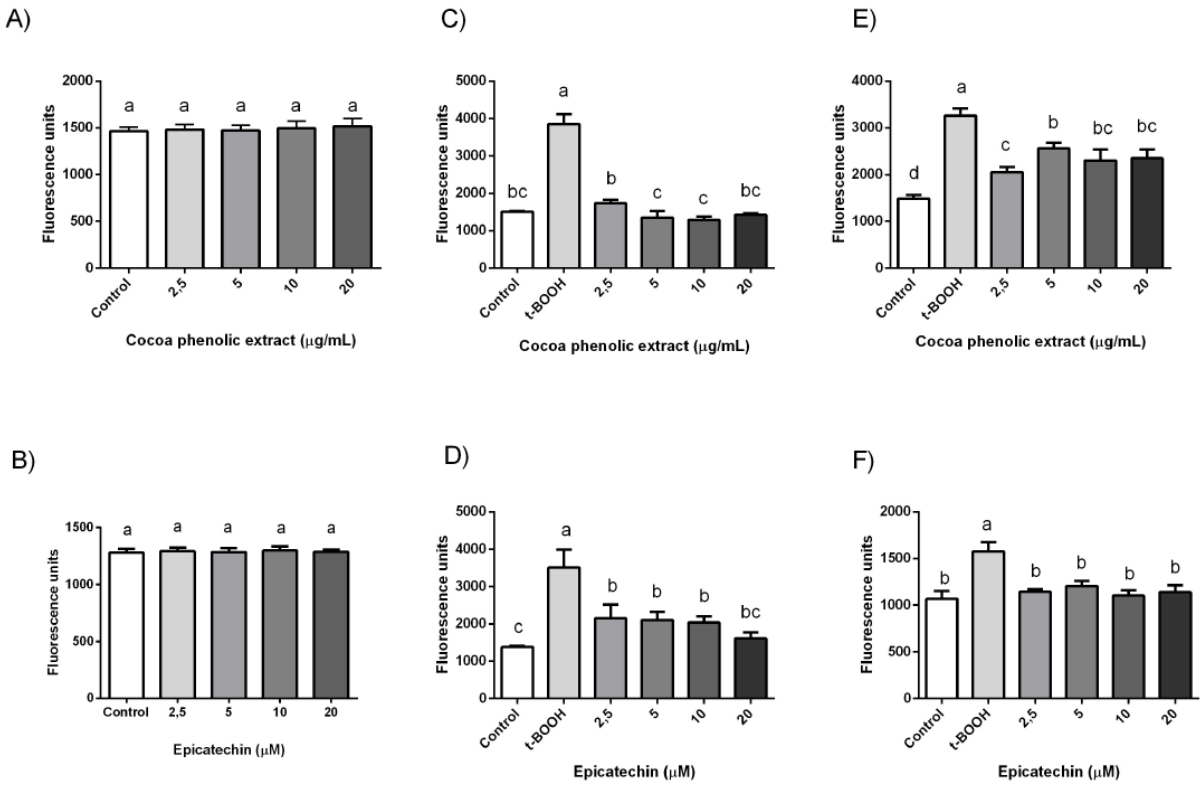

Fig. 2

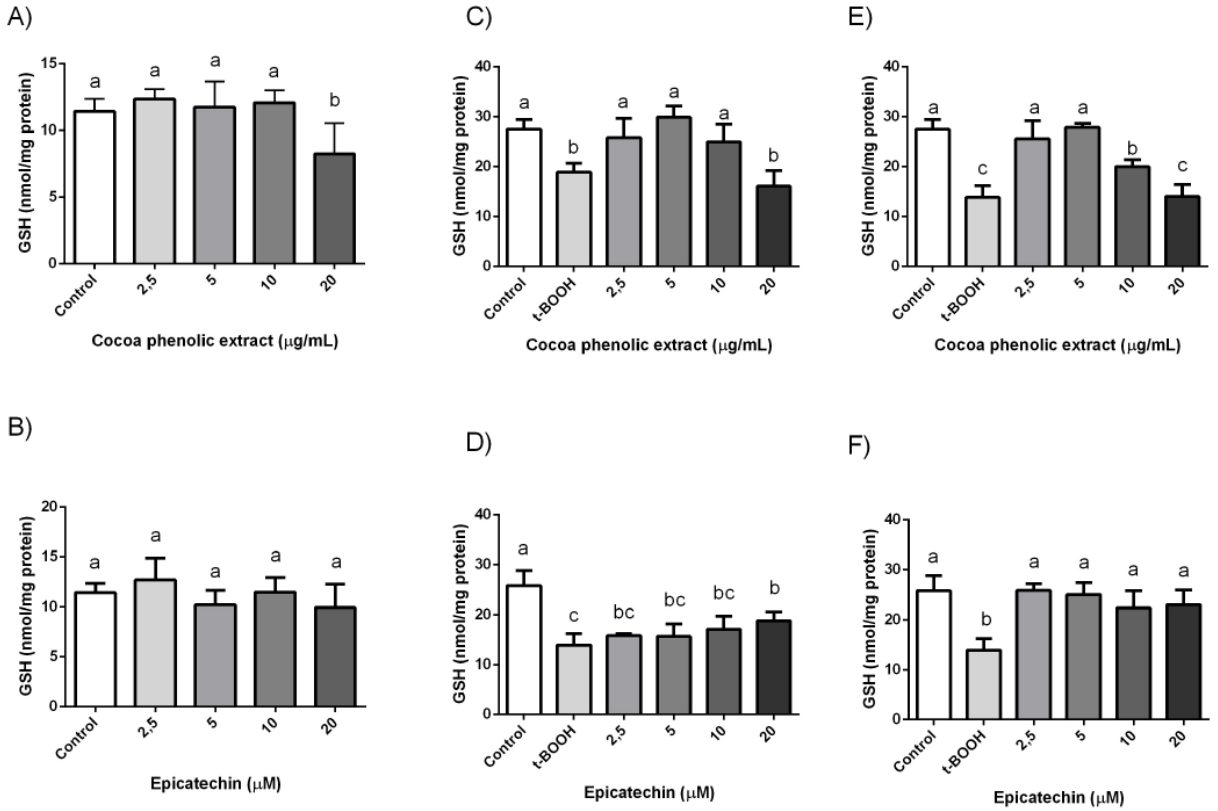
A)
C)
E)
B)
D)
F)
GSH (nmol/mg protein)
Control
t-BOOH
2,5
5
10
20
Cocoa phenolic extract (μg/mL)
Epicatechin (μM)

Fig. 3

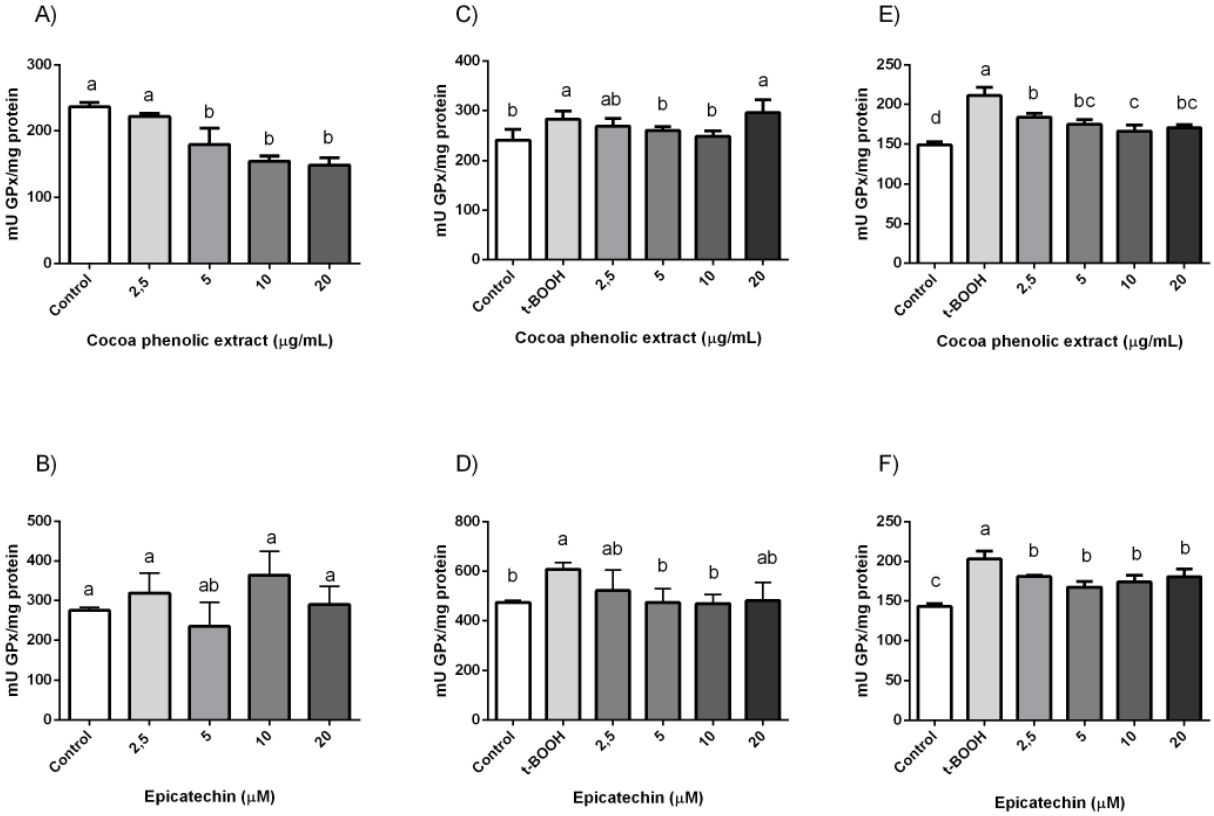
A)
C)
E)
B)
D)
F)
mU GPx/mg protein
Control
t-BOOH
2,5
5
10
20
Cocoa phenolic extract (μg/mL)
Epicatechin (μM)

Fig. 4

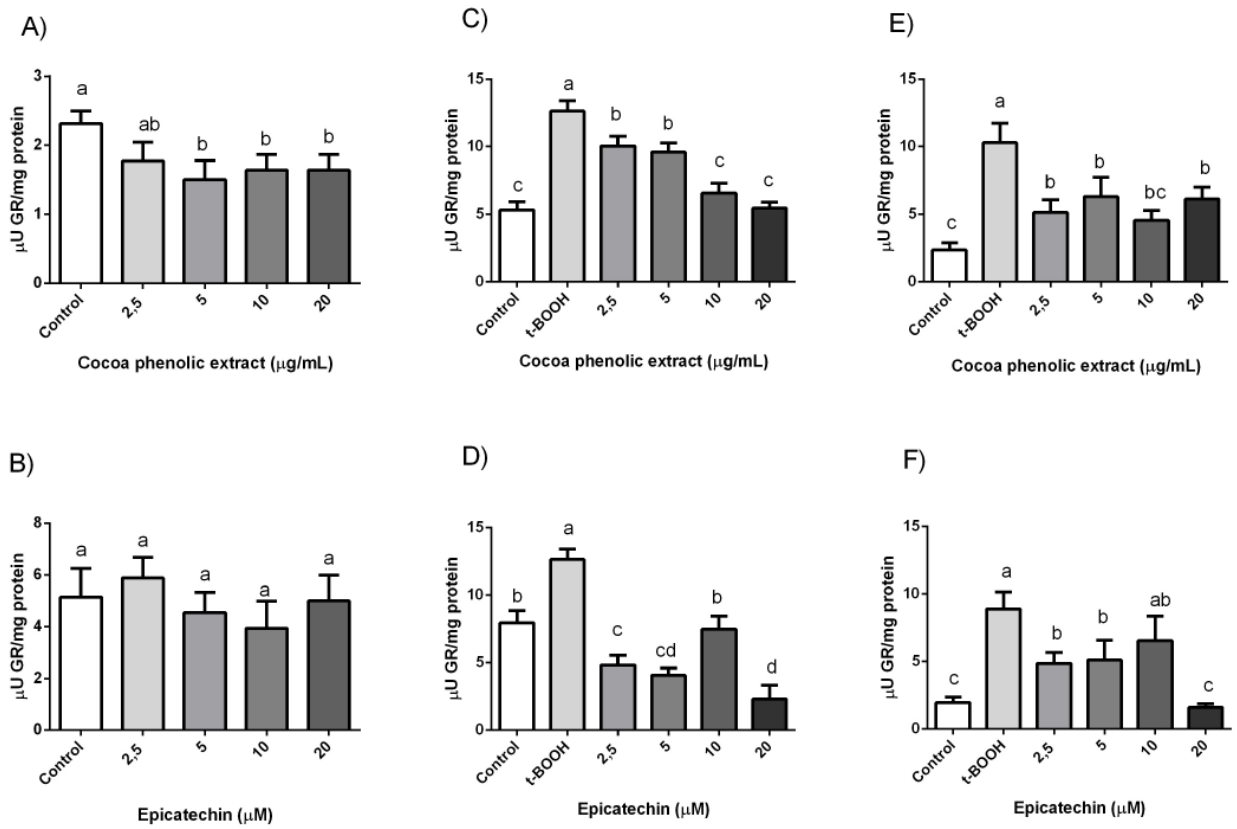
A)
C)
E)
B)
D)
F)
µU GR/mg protein
Control
t-BOOH
2,5
5
10
20
Cocoa phenolic extract (µg/mL)
Epicatechin (µM)

Fig. 5

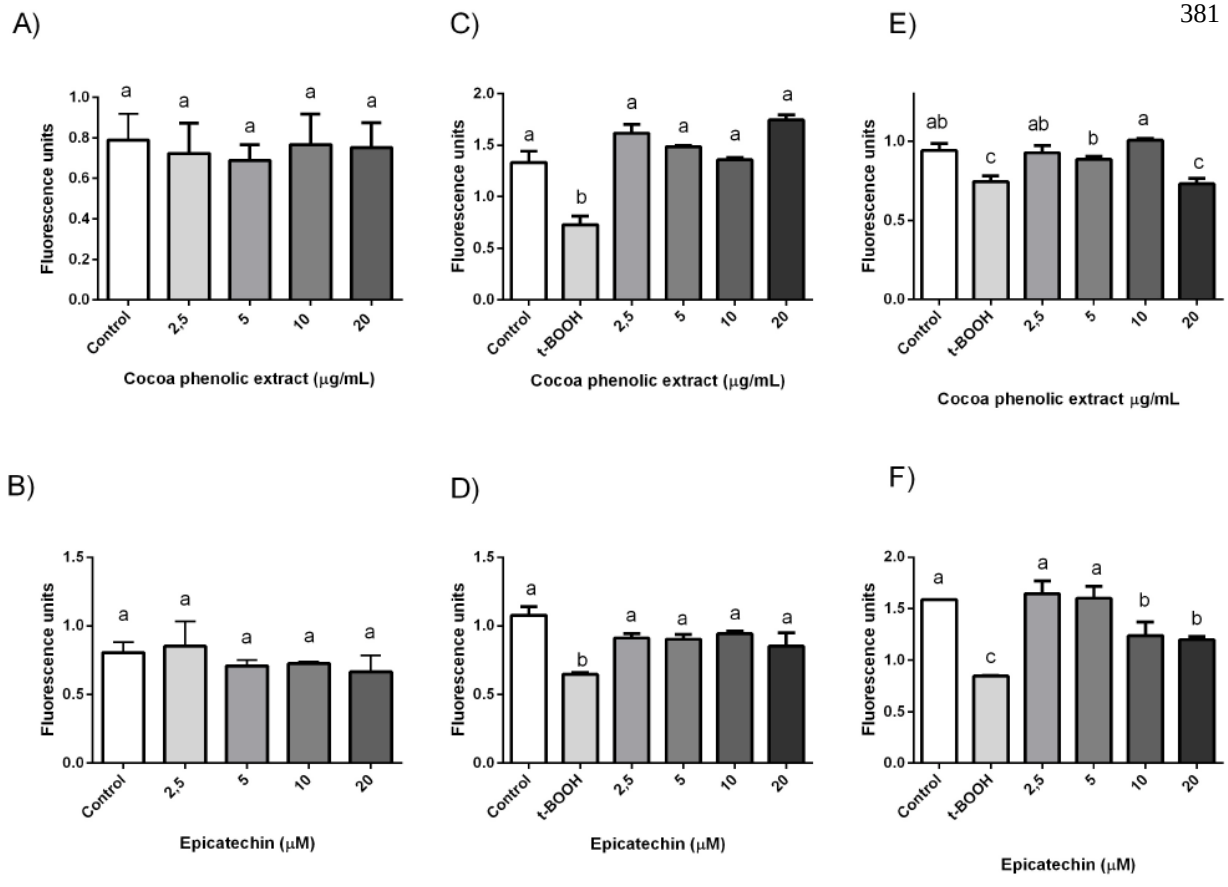
A)
C)
E)
B)
D)
F)
Fluorescence units
Control
t-BOOH
2,5
5
10
20
Cocoa phenolic extract (µg/mL)
Cocoa phenolic extract µg/mL
Epicatechin (µM)

Fig 1

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Click here to view linked References

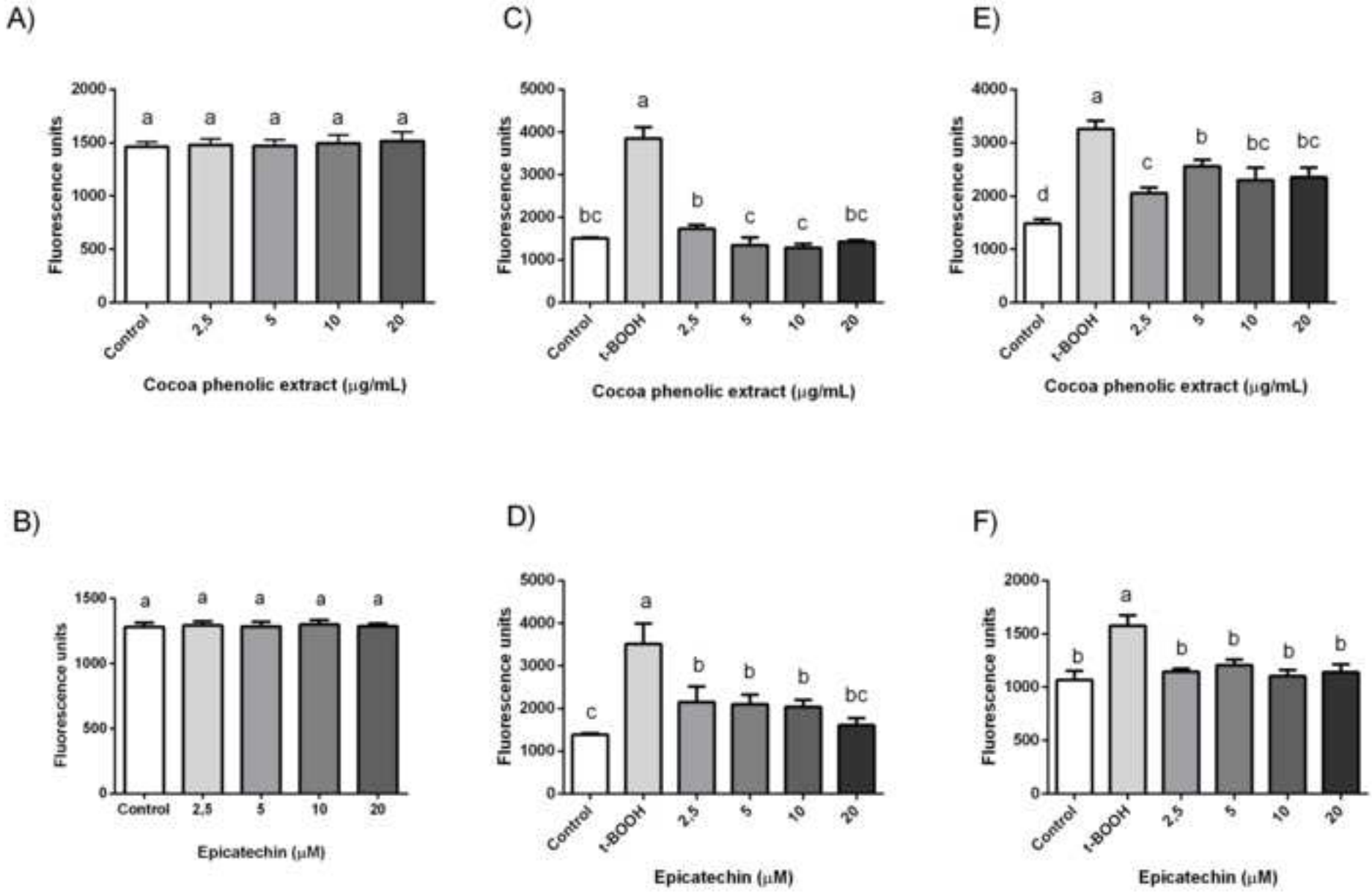

Fig 2

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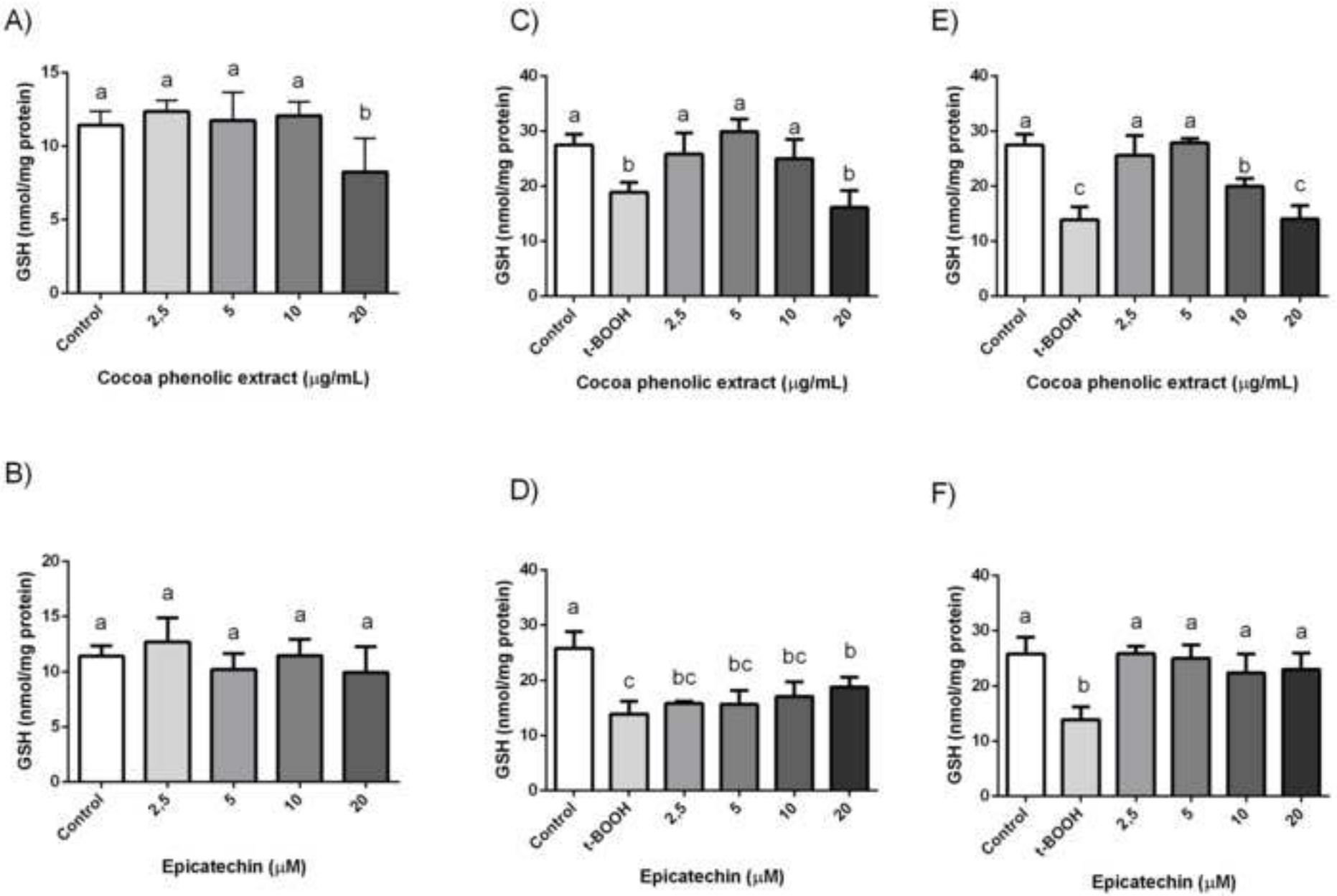

Fig 3

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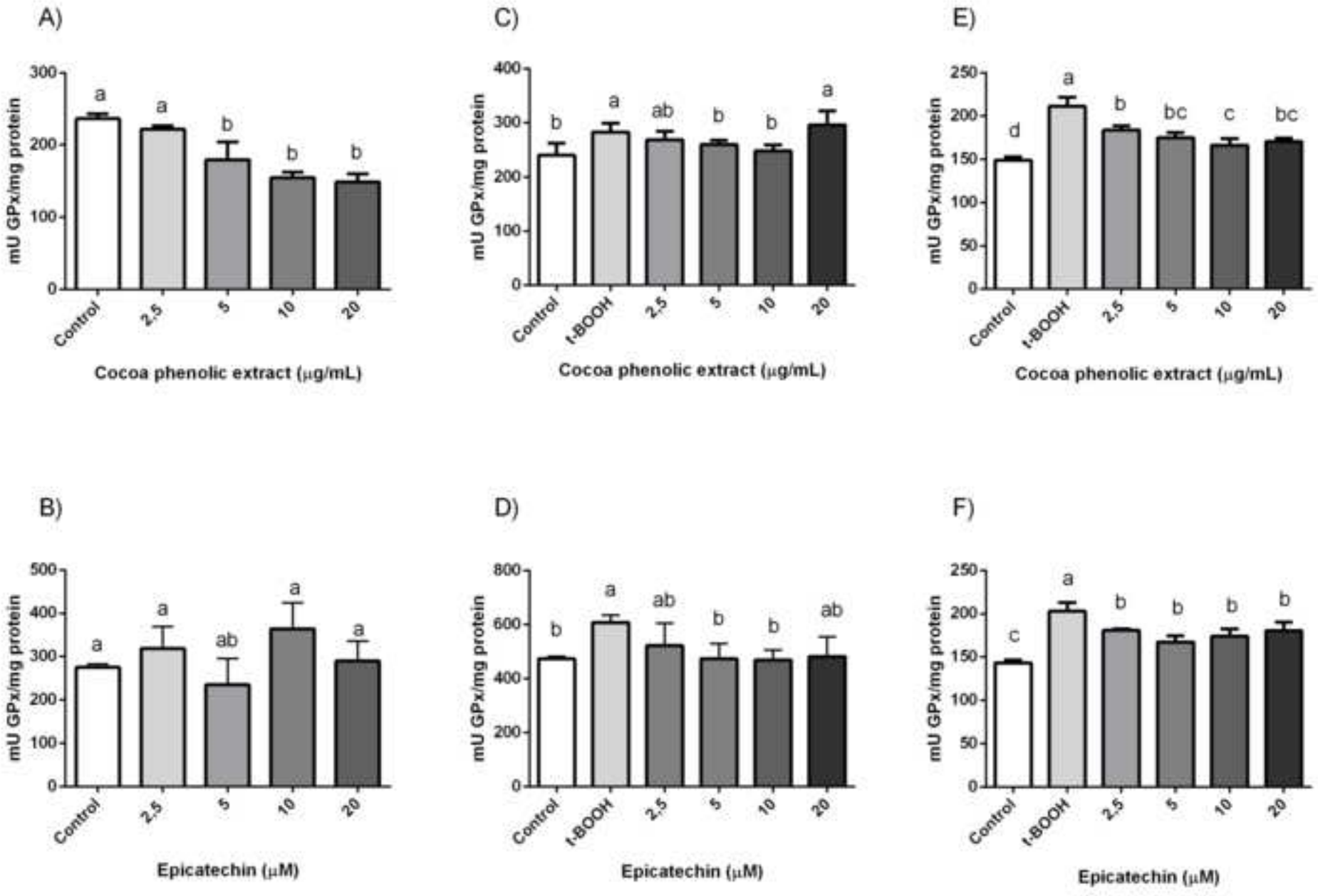

Fig 4

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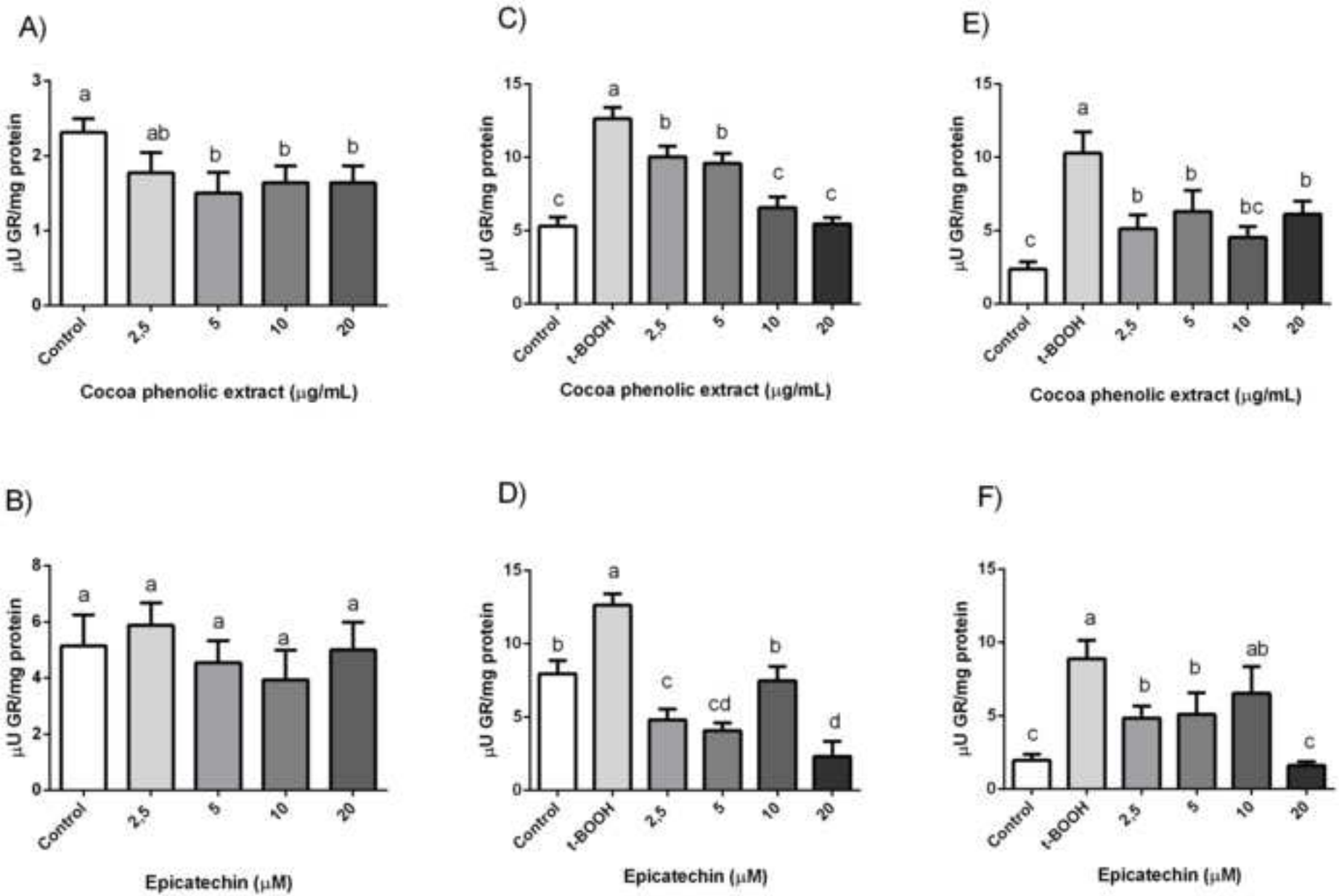

Fig 5

Click here to access/download;attachment to manuscript;Fig5.tif

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